

**Amendments to the specification:**

Please replace the Abstract at page 97, with the following abstract:

The present invention relates to phosphorylated tau protein epitopes associated with Alzheimer's disease, to protein kinases responsible for tau protein epitope phosphorylation, and to antibodies specific for the tau epitopes. Additionally, the present invention relates to pharmaceutical compositions, methods for detection, and methods of testing drugs effective in dissolving paired helical filaments associated with Alzheimer's disease.

Please amend the specification starting at page 23, line 20 to page 45, line 24 as follows:

[0176] ~~FIG.~~ FIGURE 1aA: Aminoacid sequence of tau (SEQ ID NO: 1) (isoform htau40, Goedert et al., 1989). The motifs SP, TP, IGS and CGS are highlighted.

[0177] ~~FIG.~~ FIGURE 1bB: (aA) SDS gel of tau isoforms, (bB) immunoblots of (aA) and PHF tau with the AT8 antibody. (aA) SDS gel. Lane 1, marker proteins. Lane 2: Tau from bovine brain, showing several isoforms in a mixed state of phosphorylation. Lane 3, bovine brain tau after dephosphorylation with alkaline phosphatase. Note that all isoforms shift to a lower  $M_r$ . Lanes 4 and 5: Tau from normal human brain, before and after dephosphorylation. Lanes 6-11: bacterially expressed human tau isoforms htau23, 24, 37, 34, 39, 40 (see Goedert et al., 1989, *ibid.*). These isoforms have either three or four internal repeats of 31 residues each in the C-terminal half (three: htau23, 37, 39; four: htau24, 34, 40). Near the N-terminus there can be zero, one, or two inserts of 29 residues (zero: htau23, 24; one: htau37, 34; two: htau39, 40).

[0178] (bB) Immunoblots with the AT8 antibody. Lane 1, PHF tau, showing 4-6 isoforms in the range of 60-70 kD; all of them react strongly with AT8. Lanes 2-11, same preparations as in (aA); none of the bovine or normal human tau isoforms show any reaction.

[0179] ~~FIG.~~ FIGURE 2: Phosphorylation of bacterially expressed human tau isoforms with the kinase from brain. (aA) SDS gels, (bB) immunoblots with AT8.

[0180] (aA) Lanes 1 and 2, SDS gel of htau23 before and after extract phosphorylation (note the

upward shift in  $M_r$ ) Lanes 3-10 show analogous pairs for other isoforms (htau24, 34, 39, 40).

[0181] (bB) Immunoblots of (aA) with AT8 antibody. It reacts with all tau isoforms after phosphorylation (even lanes; including htau37, not shown here).

[0182] ~~FIG.~~ FIGURE 3: Diagram of constructs K3M, K10, K19, and K17. K19 (99 residues) contains the sequence Q244-E372 (SEQ ID NO: 28) of htau23 plus an N-terminal methionine. This comprises three of the repeats (repeat 1, 3, and 4; repeat 2 is absent in htau23). K10 (168 residues) is similar, except that it extends to the C-terminus of htau23 (L441). K17 (145 residues) contains the sequence S198-E372 (assembly domain starting at the chymotryptic cleavage site, up to end of fourth repeat, but without the second repeat, plus an N-terminal methionine). K3M (335 residues) contains the N-terminal 154 residues of bovine tau4, plus the sequence R221-L441 of htau23 (without second repeat). The location of peptide S198-T220 is indicated in K17. By comparison of the constructs the epitope of AT8 must be in this region (see ~~FIG.~~ FIGURE 4).

[0183] ~~FIG.~~ FIGURE 4: Phosphorylation of htau40 and constructs K10, K17, K3M, and K19.

[0184] (aA) SDS gel. Odd lanes, htau40, K10, K17, and K3M before phosphorylation, even lanes, after phosphorylation. Note the upward shift of the bands after phosphorylation. In lane 4 there are two bands because K10 is not completely phosphorylated.

[0185] (bB) Immunoblot of (aA) with AT8. The antibody reacts only with htau40 (lane 2) and K17 (lane 6), both in the phosphorylated state, but not with K10 (lane 4) or K3M (lane 8), although these constructs are also phosphorylated and show an  $M_r$  shift.

[0186] (eC) Construct K19 before and after incubation with the kinase. Lanes 1 and 2, SDS gel; there is no  $M_r$  shift and no phosphorylation, confirmed by autoradiography (not shown). Lanes 3 and 4, immunoblot with AT8, showing no reaction. This confirms that the epitope is not in the repeat region.

[0187] ~~FIG.~~ FIGURE 5: Diagram of tryptic peptide S195-R209. The 15 residue peptide (SEQ ID NO: 29) (containing 5 serines and 1 threonine) was labeled with two radioactive phosphates at S199 and S202, as determined by sequencing.

[0188] ~~FIG.~~ FIGURE 6: Phosphorylation and antibody reactions of the D-mutant of htau23 (S199 and S202 changed into D). Lanes 1 and 2, SDS gel of htau23 before and after extract phosphorylation; lanes 3 and 4, D-mutant before and after extract phosphorylation. Note that the D-mutant runs slightly higher than htau23 (lanes 1,3), but after phosphorylation both proteins have the same position in the gel (lanes 2, 4).

[0189] Lanes 5-8, immunoblots of lanes 1-4 with AT8. The antibody reacts only with extract phosphorylated htau23 (lane 6), but neither with the unphosphorylated form (lane 5) nor with the D-mutant (lanes 7, 8), although it was phosphorylated as seen by the additional shift and autoradiography (not shown).

[0190] Lanes 9-12, immunoblots of lanes 1-4 with TAU1. This antibody reacts only with htau23 before phosphorylation (lane 9), but not with the phosphorylated form (lane 10) nor with the D-mutant (lanes 11, 12). The aspartic acid apparently mimicks a phosphorylated serine and thus masks the epitope. The minor reaction of htau23 with TAU1 in lane 10 shows that the protein is not completely phosphorylated.

[0191] ~~FIG.~~ FIGURE 7: Time course of phosphorylation of bacterially expressed human isoform htau23 with the brain kinase activity and corresponding autoradiogram.

[0192] (aA) SDS-PAGE of htau23 after incubation with the kinase between 0 and 24 hours, as indicated. The unphosphorylated protein is a single band of  $M_r=48$  kD (lane 1). Lanes 3-14 show that phosphorylation leads to a progressive shift to higher  $M_r$  with well defined intermediate stages. The even lanes (numbered 4, 6, etc. below FIG. 1bB) are observed in the presence of 10  $\mu$ m okadaic acid (OA) (labeled "+" below FIG. 1aA). The odd lanes (3, 5, etc. labeled "-") are without okadaic acid. The first stage takes about 2 hours (shift to a new  $M_r=52$  kD), the second is finished around 10 hours ( $M_r=54$  kD), the third is finished around time 24

hours ( $M_r=56$  kD); no further shift is observed during the subsequent 24 hours. Lane 2 shows a mutant that is not of significance in this context.

[0193] (**bB**) Autoradiogram of (**aA**). The quantitation of the phosphate incorporated (mol  $P_i$ /mol protein) in this experiment was as follows (-OA/+OA): 30 min (0.5/1.0), 60 min (0.7/1.4), 120 min (1.0/2.0), 10 hours (2.0/3.0), 24 hours (3.2/4.0).

[0194] ~~FIG.~~ FIGURE 8: (**aA**) SDS gel showing the time course of phosphorylation of htau23 similar to that of ~~FIG.~~ FIGURE 1-aA, but with 10  $\mu$ M okadaic acid throughout; (**bB**) immunoblot of (**aA**) with the monoclonal antibody SMI34. The antibody recognizes the protein only in the second and third stage of phosphorylation, but not in the first.

[0195] ~~FIG.~~ FIGURE 9: Binding of tau isoforms to microtubules before and after phosphorylation.

[0196] (**aA**) SDS gel of a binding experiment, illustrated for the case of the tau isoform htau40 (whose band is clearly separated from that of tubulin (T) so that both components can be shown simultaneously, without having to remove tubulin by a boiling step). The top line indicates pellets (P) or supernatants (S), with or without phosphorylation for 24 hours (+or - $P_i$ ). Lanes 1-4, 20  $\mu$ M tau protein (total concentration), phosphorylated (lanes 1, 2) or not (lanes 3, 4). The comparison of lanes 1 and 2 shows that most of the phosphorylated protein is free (S), while only a small fraction is bound to the microtubules (P). Lanes 3 and 4 show that in the unphosphorylated state about half of the protein is bound, the other half free (note also that the phosphorylated protein bands, lanes 1, 2, are higher in the gel than the unphosphorylated ones, lanes 3, 4, similar to ~~FIG.~~ FIGURE 1). Lanes 5-8, similar experiment with 15  $\mu$ M htau40. Lanes 9, 10 show the case of 10  $\mu$ M phosphorylated protein. Lanes 11-15 are for density calibration with known amounts of htau40 (15, 10, 7.5, 5, and 2.5  $\mu$ M, resp.).

[0197] (**bB**) Binding curves of htau23 and (**cC**) htau34 to microtubules before (circles) and after 24 hour phosphorylation (triangles); these curves were derived from SDS gels similar to that of ~~FIG.~~ FIGURE 3-aA. Polymerized tubulin is 30  $\mu$ M. Fitted dissociation constants  $K_d$  and

stoichiometries are as indicated. In each case the most dramatic effect is on the number of binding sites which decrease about three-fold upon phosphorylation, from around 0.5 (i.e. one tau for every two tubulin dimers) down to about 0.16 (one tau for six tubulin dimers). Note that the binding of unphosphorylated 4-repeat isoforms (such as htau34) is particularly tight ( $K_d$  round 1-2  $\mu$ M).

[0198] ~~FIG.~~ FIGURE 10: Diagram of htau40, showing the location of the 7 ser-pro motifs phosphorylated by the kinase activity. The boxes labeled 1-4 are the internal repeats involved in microtubule binding; the second is absent in some isoforms (e.g. htau23). The two shaded boxes near the N-terminus are inserts absent in htau23 and htau24 so that these molecules have only 6 ser-pro motifs. The following radioactive tryptic peptides were found:

[0199] 24-49: KDQGGYTMHQOQEGOTDAGLKES<sub>p</sub>PLQ (SEQ ID NO: 31)

[0200] 191-209: SGDRGYSS<sub>p</sub>PGS<sub>p</sub>PGTPGSR (SEQ ID NO: 32)

[0201] 231-240: TPPKS<sub>p</sub>PSSAK (SEQ ID NO: 33)

[0202] 396-405: SPVVSGDTS<sub>p</sub>PR (SEQ ID NO: 34)

[0203] 385-405: TDHGAEIVYKS<sub>p</sub>PVVSGDTS<sub>p</sub>PR (SEQ ID NO: 35)

[0204] 407-428: HLSNVSSTGSIDMVDS<sub>p</sub>PQLATL (SEQ ID NO: 36)

[0205] 260-266: IGS<sub>p</sub>TENL (SEQ ID NO: 37)

[0206] ~~FIG.~~ FIGURE 11: Binding of htau34 to microtubules, before (circles) and after phosphorylation for 90 min (stage 1, triangles). The reduction in binding capacity is very similar to that after 24 hours phosphorylation (compare ~~FIG.~~ FIGURE 9bB).

[0207] ~~FIG.~~ FIGURE 12: SDS-PAGE and immunoblots of tau protein from Alzheimer and

normal human brain with antibodies SMI33, SMI31, and SMI34.

[0208] (aA) Lane 1, SDS-PAGE of tau protein from a normal human control brain, showing 5-6 bands between  $M_r$  55 and 65 kD (somewhat lower than the PHF tau of lane 3). Lane 2, normal human tau after phosphorylation with kinase activity, resulting in an upward shift of all bands. Lanes 3, 4, immunoblot of PHF tau with antibody 5E2 which recognizes all tau isoforms independently of phosphorylation (Kosik et al., Neuron 1 (1988), 817-825). Lane 3, PHF tau as isolated from an Alzheimer brain; lane 4, after dephosphorylation with alkaline phosphatase. Note that the bands of the dephosphorylated protein are shifted down on the gel.

[0209] (bB) Immunoblot of (aA) with SMI33. The antibody recognizes normal human tau (lane 1), and PHF tau after dephosphorylation (lane 4).

[0210] (cC) Immunoblot of (aA) with SMI31. Note that the antibody recognizes normal human tau after phosphorylation, and PHF tau in its natural state of phosphorylation (lanes 2, 3).

[0211] (dD) Immunoblot of (aA) with SMI34. This antibody recognizes normal human tau only after phosphorylation (lane 2), and PHF tau (lane 3).

[0212] ~~FIG.~~ FIGURE 13: Time course of phosphorylation of bacterially expressed human isoform htau23 (similar to previous figure) and immunoblots with antibodies SMI33, SMI31, SMI34, TAU1, and AT8.

[0213] (aA) SDS-PAGE, phosphorylation times 0-24 hours, showing the successive  $M_r$  shifts. (~~b~~-fB-F) Immunoblots with SMI31, SMI34, SMI33, TAU1, and AT8. Antibodies SMI33 and TAU1 recognize htau23 fully up to the end of stage 1 (2 hours), but the epitope becomes blocked during the second stage. Antibodies SMI31, SMI34, and AT8 are complementary in that they recognize the protein only in the second and third stage of phosphorylation.

[0214] (~~g~~-hG-H) Immunoblot of htau34 with SMI33 and SMI310 which recognize the protein from the stage 2 phosphorylation onwards, similar to SMI31.

[0215] ~~FIG.~~ FIGURE 14: SDS-PAGE of tau and several constructs, and immunoblots with the antibodies SMI33, SMI31, and SMI34.

[0216] (aA) SDS-PAGE. Lanes 1 and 2: Construct K10 before and after phosphorylation with the kinase for 24 hours. Lanes 3 and 4: Construct K17 before and after phosphorylation. Lanes 5 and 6: Construct K19 before and after phosphorylation. All constructs except K19 show a shift upon phosphorylation. With K10 one observes three shifted bands, with K17 there is only one shifted band.

[0217] (bB) Immunoblot of (aA) with SMI33: The antibody recognizes only K17 in the unphosphorylated form (lane 3), suggesting that the epitope lies before the repeats.

[0218] (cC) Immunoblot of (aA) with SMI34. The antibody recognizes K10 and K17 in the phosphorylated form (only top bands, lanes 2, 4). The antibody does not recognize K19 (the repeat region), but requires sequences on both the N-terminal and C-terminal side of the repeats. The epitope is therefore non-contiguous (conformation-dependent).

[0219] (dD) Immunoblot of (aA) with SMI31. The antibody recognizes only the top band of the phosphorylated K10 (lane 2), suggesting that the epitope lies behind the repeat region.

[0220] ~~FIG.~~ FIGURE 15: (A-G) Diagram of point mutants of htau40 and htau23.

[0221] ~~FIG.~~ FIGURE 16: SDS gel of htau40 and the point mutants of ~~FIG.~~ FIGURE 15, and immunoblots with antibodies SMI33, SMI31, and SMI34.

[0222] (aA) Lanes 1-8, SDS gel of htau40 and its mutants KAP235, KAP396, and KAP235/396 in the unphosphorylated and phosphorylated form (+). In each case phosphorylation leads to an upward shift in the SDS gel.

[0223] (b) Blot of (aA) with SMI33. The antibody response is strongly reduced when S235 is

mutated, both in the dephosphorylated and phosphorylated state (lanes 3+4, 7+8). This indicates that the (dephosphorylated) first KSP motif is part of the epitope of SMI33. When S396 is mutated to A the behavior is similar to the parent molecule, i.e. strong antibody response in the dephosphorylated state, no reaction in the phosphorylated state, so that S396 does not contribute to the epitope of SMI33.

[0224] (eC) Blot of (aA) with SMI31. The antibody recognizes htau40 and all mutants in the phosphorylated form (lanes 2, 4, 6, 8). This shows that phosphorylation of the two KSP motifs is not the main determinant of the epitope.

[0225] (dD) Blot of (aA) with SMI34. The reaction is similar to SMI31 but more pronounced, again indicating that the two KSP motifs are not essential.

[0226] FIG. FIGURE 17:: Deletion mutants of tau and their antibody response. (aA) SDS gel of constructs containing only two repeats (K5-K7) or one repeat (K13-K15), before and after phosphorylation. (bB) Immunoblot of (aA) with SMI34. Note that the antibody recognizes all phosphorylated proteins (K7 only weakly). (cC) Immunoblot of (aA) with SMI31. Note that the antibody recognizes the phosphorylated two-repeat molecules (K5-K5), but not the one-repeat molecules (K13-K15). Lanes 7 and 8 show htau40 as a control. (dD) SDS gel of constructs K2, K3M, and K4, before and after phosphorylation. (eE) Blot of (dD) with SMI34, recognizing only K4 phosphorylated. (fF) Blot of (dD) with SMI31, recognizing only K2 phosphorylated.

[0227] FIG. FIGURE 18:(A-M) Diagram of htau40 and various mutants used in this study.

[0228] FIG. FIGURE 19: Diagram of tau isoforms and constructs used in studies on tau dimerization and oligomerization

[0229] (dD) T8R-1,553 residues, MW 57743, derived from htau40 (see below). It has two inserts near the N-terminus (29 residues each, hatched), a repeat domain of four repeats (numbered 1-4) which is duplicated with a small spacer in between.



[0230] (~~b~~B) T8R-2,511 residues, MW 53459; it lacks the N-terminal inserts, but has the four repeats duplicated.

[0231] (~~e~~C) T7R-2,480 residues, MW 50212; similar to T8R-2, but without the second repeat sequence in the first repeat domain.

[0232] (~~d~~D) Htau40,441 residues, MW 45850, the largest of the six human tau isoforms (Goedert et al.), with two N-terminal inserts and a repeat domain containing four repeats.

[0233] (~~e~~E) Htau23,352 residues, MW 36760, the smallest of the human tau isoforms, without the N-terminal repeats and only three repeats.

[0234] (~~f~~F) K11,152 residues, MW 16326, a repeat domain with four repeats plus a short tail.

[0235] (~~g~~G) K12,121 residues, MW 13079, a repeat domain with three repeats plus a short tail.

[0236] ~~FIG.~~ FIGURE 20: SDS PAGE (4-20%) and gel chromatography of tau constructs and cross-linked products. Gels a and c were run in reducing conditions (3 mM DTT in sample buffer), gel b in non-reducing conditions (except lane 1 with 3 mM DTT in sample buffer).

[0237] (~~d~~D) Constructs T8R-1, Htau23 and K12. Molecular weight markers are given on the left.

[0238] (~~b~~B) Construct K12 and cross-linked products. Cross-linking occurs spontaneously in the absence of DTT; it can be prevented by DTT, or induced by addition of PDM or MBS.

Aggregation products are labeled on the right (~~monomers~~, dimers, trimers, tetramers etc.).

[0239] (~~e~~C) Silver stained SDS gel of a Superose 12 gel filtration run of K12 cross-linked by PDM. The dimers (top band) elute before the monomers. Fractions 16 and 17 were used for electron microscopy.

[0240] (~~d~~D) Elution profile of Superose 12 gel filtration of construct K12 monomers and dimers

cross-linked with PDM. The elution positions of calibration proteins are plotted against their effective hydrated Stokes radii on a logarithmic scale (right axis).

[0241] (eE) CD spectrum of construct K12 (8 mg/ml in 40 mM HEPES pH 7.2, path length 0.01 mm). There is no significant  $\alpha$ -helical or  $\beta$ -sheet structure. Similar spectra are obtained with other constructs as well as with full length tau.

[0242] ~~FIG.~~ FIGURE 21: Synthetic paired helical filaments from construct K12.

[0243] (aA) A tangle of synthetic PHFs from K12 (crossover period of .apprxeq.70-75 nm indicated by arrowheads). The construct was expressed and purified by the methods described previously (Steiner et al.). It was dialysed against 0.5 M Tris-HCl, with pH values between 5.0 and 5.5. The solution was negatively stained with 2% uranyl acetate.

[0244] (bB) and (eC) Single fibers of synthetic paired helical filaments made from construct K12. Note the crossover repeats (arrowheads) and the rod-like particles of lengths around 100 nm (c, middle). Bar=100 nm.

[0245] ~~FIG.~~ FIGURE 22 (A-C): Synthetic paired helical filaments from K12 dimers cross-linked with PDM and negatively stained with 1% phosphotungstic acid (micrographs provided by M. Kniel). Bar=100 nm.

[0246] ~~FIG.~~ FIGURE 23: Paired helical filaments from Alzheimer brain (micrographs provided by Dr. LichtenbergKraag).

[0247] (aA) PHFs from neurofibrillary tangles prepared after Wischik et al., stained with 1% phosphotungstic acid. This preparation contains homogeneous long filaments which still retain their pronase sensitive "fuzzy coat." The crossover repeat is 75-80 nm, the width varies between a minimum of about 10 nm and a maximum of 22 nm.

[0248] (bB) PHFs prepared after Greenberg & Davies. This preparation results in soluble

filaments of shorter length than in (aA) and is more heterogeneous. (1) is a paired helical filament with a 72 nm repeat and a width varying between 8 and 18 nm; (2) is a straight filament of 8 nm width; (3) is a twisted filament with a particularly wide diameter (up to 25 nm); (4) is a straight filament with a wide diameter (18 nm); (5) is a twisted rod-like particle about 80 nm long, equivalent to about one crossover period. In many cases the particles appear to have broken apart across the filament, e.g. the two rods labeled (4), the twisted filament of (3) and the short stub to the right of it, or the two straight rods above particle (3). Bar=100 nm.

[0249] ~~FIG.~~ FIGURE 24: Electron micrographs of tau isoform htau23 and construct T8R-1 prepared by glycerol spraying and metal shadowing

[0250] (aA) monomers of htau23,

[0251] (bB) dimers of htau23,

[0252] (cC) monomers of T8R-1,

[0253] (dD) folded forms of T8R-1 (hair-pin folds showing intramolecular antiparallel association),

[0254] (eE) dimers of T8R-1. For lengths see Table 1 and ~~FIG.~~ FIGURE 7. Interpretative diagrams are shown on the right. Bar=50 nm.

[0255] ~~FIG.~~ FIGURE 25 (A-H): Length histograms of tau constructs and dimers.

[0256] ~~FIG.~~ FIGURE 26: Electron micrographs of constructs K11 and K12.

[0257] (aA) Monomers of K11,

[0258] (bB) dimers of K11

[0259] (eC) tetramers of K11 formed by longitudinal association of two dimers.

[0260] (eC) Monomers of K12,

[0261] (dD) dimers of K12,

[0262] (eE) tetramers of K12. Bar=50 nm.

[0263] ~~FIG.~~ FIGURE 27: (aA) K12 dimers cross-linked by PDM (i.e. Cys322 to Cys322);

[0264] (B) K12 dimers cross-linked by MBS (i.e. Cys322 to nearby Lys). Bar=50 nm.

[0265] ~~FIG.~~ FIGURE 28: Antibody labeling of htau23, K12 and cross-linked products thereof.

[0266] (aA) htau23 dimers with an antibody at one end (left) and with an antibody at each end (right) demonstrating the antiparallel dimerization of htau23;

[0267] (bB) K12 dimers with an antibody at one end (left), with antibodies at both ends (middle) and presumable tetramers with antibodies at the free ends (right) indicating that this type of association blocks the epitope;

[0268] (eC) K12 dimers cross-linked with PDM, with an antibody at one end (left), with antibodies at each end (middle) and a tetramer with antibodies at the free ends (right);

[0269] (dD) K12 dimers cross-linked by MBS with an antibody at one end (left), with antibodies at each end (middle) and a tetramer with antibodies at the free ends (right). Bar=50 nm.

[0270] ~~FIG.~~ FIGURE 29 (A-G): Time course of phosphorylation of htau40 by GSK3 and immune response. (1A) SDS-PAGE of htau40 after incubation with the kinase between 0 and 20 hours at 37.degree. C. The minor lower band in lane 1 is a fragment. Note the progressive shift to higher Mr values, similar to the effects of brain extract and MAP kinase. (2B) Autoradiography.

(3C) Immunoblot with the antibody TAU1 whose reactivity is lost after .apprxeq.2 h (following the phosphorylation of S199 and S202). (D) Immunoblot with antibody AT-8. (5E) Immunoblot with antibody SMI34 (conformation sensitive and against phosphorylated Ser). (6F) Blot with SMI31 (epitope includes phosphorylated S396 and S404). (7G) Blot with antibody SMI33 which requires a dephosphorylated S235. There are some differences with respect to phosphorylation by MAP kinase or the brain extract. The SMI33 staining persists for a long period, suggesting that Ser235 is only slowly phosphorylated by GSK3. The staining of SMI31 appears very quickly, before. that of ATS or SMI34, showing that S396 and S404 are among the earliest targets of GSK3.

[0271] FIG. FIGURE 30 (A-B): Mobility shift of htau23 versus mutant htau23/A404 upon phosphorylation with GSK3. Top, SDS gel, bottom, autoradiogram. Lanes 1-3, htau23 unphosphorylated and phosphorylated for 2 or 20 hours. Note the pronounced shift and the clear incorporation of phosphate. Lanes 4-6, mutant Ser404-Ala, unphosphorylated and phosphorylated for 2 and 20 hours. The shift after 2 hours is much smaller and the degree of phosphorylation much lower. This shows that the first strong shift and phosphorylation is at Ser404, similar as with MAP kinase and the brain extract kinase activity.

[0272] FIG. FIGURE 31: Diagrams of tau constructs. Top, AP17, a derivative of htau23 with all Ser-Pro or Thr-Pro motifs altered into Ala-Pro. Middle, AP11, only Ser-Pro motifs changed into Ala-Pro. Bottom, K18, only 4 repeats of tau (derived from htau40).

[0273] FIG. FIGURE 32: Copolymerization of MAP kinase and GSK3 with porcine brain microtubules. (aA) SDS gel of microtubule purification stages. Ex=brain extract, supernatant after first cold spin. S=supernatant of first hot spin=tubulin and MAPs not assembled into microtubules after warming to 37.degree. C.; P=pellet of redissolved microtubules. The other lanes (S, P) show two further cycles of assembly and disassembly by temperature shifts (last pellet of microtubule protein was concentrated). (bB) Blot with anti-MAP kinase, showing mainly the p42 isoform and some of the p44 isoform. (cC) Blot with anti-GSK3B; note that this antibody shows some cross-reactivity with GSK3α. (dD) Blot with anti-GSK3α. The blots show that both kinases and their isoforms co-purify with the cycles of microtubule assembly.

[0274] ~~FIG.~~ FIGURE 33: (aA) Identification of GSK3 $\alpha$  and  $\beta$  in normal and Alzheimer brain extracts. M=markers, lane 1, SDS gel of normal brain extract, lane 2, immunoblot with anti-GSK3 $\alpha$ ; lane 3, immunoblot with anti-GSK3  $\beta$  (with some cross-reactivity to  $\alpha$ ). Lanes 4 and 5, same blots with Alzheimer brain extracts. (B) Identification of GSK3 in association with PHF from Alzheimer's tau.

[0275] ~~FIG.~~ FIGURE 34: Binding curves of htau23 to microtubules (made from 10  $\mu$ M tubulin in the presence of 20  $\mu$ M taxol). Top curve (squares), htau 23 unphosphorylated. Middle (circles), htau23 phosphorylated with GSK3, showing a comparable stoichiometry as the unmodified tau protein (saturating 0.6 per tubulin dimer). Bottom curve (triangles), control of htau23 phosphorylated with the brain kinase activity, showing a pronounced decrease in stoichiometry. The solid lines show the best fits assuming independent binding sites.

[0276] ~~FIG.~~ FIGURE 35: (aA) Diagram of htau23 and point mutants used in this invention. (bB) Binding curves of htau23 and its point mutants to microtubules, unphosphorylated and phosphorylated with brain extract. The top and bottom curves show unphosphorylated and phosphorylated wild type htau23, the other curves are after phosphorylation. Mutants are (from top to bottom): Ser262-Ala, Ser235-Asp/Ser396-Asp, Ser404-Ala, Ser202-Ala. The mutation at Ser262 nearly eliminates the sensitivity of the tau-microtubule interaction to phosphorylation. These curves were derived from quantitating SDS gels by densitometry (see Example 6). Polymerized tubulin is 30  $\mu$ M. The fitted stoichiometries  $n$  ( $=\text{tau}/\text{tubulin dimer}$ ) and binding constants  $K_d(\mu\text{M})$  are:

[0277] htau23wt non-phos. ( $n=0.49$ ,  $K_d=2.5$ ); A262 phos. ( $n=0.45$ ,  $K_d=5.3$ ); D235/D396 phos. A202 phos. ( $n=0.31$ ,  $K_d=9.4$ ); htau23wt phos. ( $n=0.16$ ,  $K_d=4.9$ ).

[0278] ~~FIG.~~ FIGURE 36: Binding curves of htau40 to microtubules. Top, unphosphorylated htau40 (triangles); middle, htau40 phosphorylated with MAP kinase (circles); bottom, htau40 phosphorylated with brain extract (squares). Fitted dissociation constants  $K_d$  and stoichiometries are as indicated.

[0279] **FIG. FIGURE 37:** (aA) Diagram of total mutant AP18. All Ser-Pro Thr-Pro are replaced by Ala-Pro. In addition, Ser262 and 356 are mutated into Ala. In the mutant AP17 Ser262 and Ser356 remain unchanged. (bB) Binding curves of htau 23 and the "total" mutants AP17 and AP18 to microtubules without or with phosphorylation by brain extract. Top, unphosphorylated htau23 (filled triangles); middle, phosphorylated AP18 (circles), the two bottom curves are phosphorylated AP17 (open squares) and htau23 (open triangles). The difference in behavior between AP17 and AP18 is due to the phosphorylation of Ser262 in AP17. Fitted stoichiometries and binding constants are:

[0280] htau23wt non-phos. ( $n=0.49$ ,  $K_d=2.5$ ); AP18 phos ( $n=0.48$ ,  $K_d=6.1$ ); AP17 phos ( $n=0.18$ ,  $K_d=6.6$ ); htau23wt phos. ( $n=0.16$ ,  $K_d=4.9$ ).

[0281] **FIG. FIGURE 38:** Preparation of the kinase from porcine brain by chromatographic steps. (aA) Mono Q HR 10/10 FPLC. The phosphorylation of recombinant human tau 34 and construct AP17 is shown on the y-axis as moles  $P_i$  transferred per mole of tau. Fractions which decrease the binding of tau to MT elute around fraction 12, 20 and 30, the peaks around fractions 20 and 30 being the most effective. (bB) Fractions 28-32 from Mono Q were gel filtrated on a Superdex G-75 HiLoad 16/60 column. The column was calibrated with standard proteins as shown by the filled symbols: Ribonuclease, 14 kDal; chymotrypsinogen A, 25 kDal; ovalbumin, 43 kDal; bovine serum albumin, 67 kDal. Molecular weight is indicate on the right y-axis on a logarithmic scale. The phosphorylation of htau34 and construct K18 is shown on the left y-axis. The highest activity elutes at a Mr of approx. 35 kDal. (cC) Fractions 17-23 from the gel filtration column were pooled and rechromatographed on a Mono Q HR 5/5 column. Fraction 10 was used for binding studies. (dD) SDS-gel showing the main purification stages. M: Marker proteins; lane 1, whole brain extract, lane 2, Mono Q HR 10/10 FPLC, fraction 30; lane 3, Superdex gel filtration, fraction 22; lanes 4-5, Mono Q HR 5/5 FPLC, fractions 10 and 9. Lane 5 shows the purified 35 kDal band and a trace at 41 kDal.

[0282] **FIG. FIGURE 39:** SDS gel and in-gel assay of kinase activity (for details see Example 11). (aA) 7-15% silver stained SDS gel of fractions 9-11 (lanes 1-3) of second Mono Q run (see

FIG. 38c). (bB) Autoradiogram of an in-gel experiment, with tau construct K9 (=four repeats plus C-terminal tail of tau) in the gel and 5  $\mu$ l each of fractions 9-11 (lanes 1-3). (eC) Autoradiogram of control gel containing no tau protein and showing no autophosphorylation of the Mono Q fractions. Note that specific kinase activities are difficult to quantify from these gels since the renatured protein tends to diffuse out of the gels; this is especially true of the 35 kDal band.

[0283] ~~FIG.~~ FIGURE 40: Effect of phosphorylation of tau by 35 kDal kinase on gel shift and microtubule binding. (aA) SDS gel of htau23 and constructs phosphorylated by several kinases. M, marker proteins. Lanes 1 and 2, htau23 without and with phosphorylation by 35 kDal kinase. Lanes 3 and 4, same experiment with point mutant htau23(Ser409-Ala) (no shift); lanes 5 and 6, point mutant htau23(Ser416-Ala) (only part of the protein phosphorylated, but otherwise same shift as in lane 2); lanes 7 and 8, point mutant htau23(Ser404-Ala) (same shift as lanes 2 and 6). The mutants show that the 35 kDal kinase induces a shift by phosphorylating Ser409. Note that Ser404 is the target of MAP kinase, Ser416 of CaM kinase (Steiner et al., 1990, *ibid.*), and Ser409 and Ser416 of PKA, each of which induces a shift. Lanes 9-11 show a comparison of the shifts induced in htau23 by the different kinases (CaM kinase, PKA and MAP kinase). The shifts induced by PKA (lane 10) is the same as that of the 35 kDal kinase, and that MAP kinase produces by far the largest shift, typical of the Alzheimer-like state of tau. The bars on the right indicate the shift level; from bottom to top, unphosphorylated htau23 (control), CaM kinase shift level, PKA shift level, MAP kinase shift level. All shift sites are near the C-terminus. (bB) Binding curves of htau23 and the mutants Ser262-Ala to microtubules without or with phosphorylation by the 35 kDal kinase (Mono Q fraction 10, 20 hours). Top, unphosphorylated htau23 (open circles,  $n=0.49$ ,  $K_d=2.5 \mu\text{M}$ ); middle, phosphorylated mutant (squares,  $n=0.44$ ,  $K_d=11.6 \mu\text{M}$ ); bottom, phosphorylated htau23 (filled circles,  $n=0.21$ ,  $K_d=8.8 \mu\text{M}$ ). In the absence of Ser262 the reduction in stoichiometry is 0.05; with phosphorylated Ser262 it is 0.28.

[0284] ~~FIG.~~ FIGURE 41: Diagram of htau40, highlighting the first microtubule-binding repeat (SEQ ID NO: 30) and the Ser262 that is important for microtubule binding.

[0285] ~~FIG.~~ FIGURE 42: 1. Dephosphorylation ("dephos.") of p32-marked htau40



("ht40.sup.32P") with different PPases. Autodiagraphs of 7-15% SDS gradient gels. ~~FIG.~~  
FIGURE 1: Autoradiographs of 7-15% SDS gradient gels.

[0286] A. Dephos. with PP2a H-isoform (10 µg/ml) Lane 1: ht40P before dephos. Lane 2: 10 min dephos. Lane 3: 30 min. dephos. Lane 4: 120 min dephos.

[0287] B. Dephos. with PP2a M-isoform (10 µg/ml), Lanes 1-4: see A.

[0288] C: Dephos. with PP2a L-isoform (10 µg/ml), Lanes 1-4: see A.

[0289] D: Dephos. with catalytic subunit of PP1 (500 U/ml), Lanes 1-4: see A.

[0290] ~~FIG.~~ FIGURE 43: 2. Dephosphorylation with PP2a-H: disappearing of phosphorylation dependent antibody epitopes

[0291] A. SDS-PAGE (7-15%). Lane 1: ht40P before dephos. Lane 2: 10 min dephos. Lane 3: 30 min. dephos. Lane 4: 120 min dephos. Lane 5: 5 h dephos. Lane 6: 16 h dephos.

[0292] B. Autoradiographs

[0293] C. Immunoblot AT-8

[0294] D. Immunoblot Tau-1A

[0295] E. Immunoblot SMI-33

[0296] ~~FIG.~~ FIGURE 44: Kinetics of dephos. with PP2a-H

[0297] ~~a~~A. time course of dephos. of ht40P with different concentrations of PP2a

[0298] ~~b~~B. variation in the ht40P-concentration: Michaelis-Menten-Diagramm.

[0299] ~~FIG.~~ FIGURE 45: Preparation of the 70 kDal kinase which phosphorylates the two IGS motifs and the two CGS motifs of tau protein (Serines 262, 293, 324, 409). The kinase strongly reduces the affinity of tau for microtubules.

[0300] (aA) Chromatography on S-Sepharose. Kinase activity elutes at 250 mM NaCl.

[0301] (bB) Chromatography on heparin agarose. Kinase activity elutes at 250 mM NaCl.

[0302] (eC) Gel filtration on Superdex G-75. Kinase activity elutes at 70 kDal.

[0303] ~~FIG.~~ FIGURE 46: Time course of phosphorylation of htau40 with cdk2/cyclin A. Lanes 1-9 correspond to time points 0, 10, 30, 90 min, 3, 6, 10, 24 hours, and 0 min (the 0 min lanes are the control).

[0304] (aA) SDS polyacrylamide gel electrophoresis, showing the shift of the protein upon phosphorylation.

[0305] (bB) Autoradiogram showing increasing incorporation of phosphate.

[0306] (eC) Immunoblot with TAU-1 antibody which recognizes only unphosphorylated Ser199 and Ser202.

[0307] (dD) Immunoblot with AT-8 antibody which recognizes these two serines in a phosphorylated state, as well as Alzheimer tau.

Please replace the paragraph on page 59, lines 21-32 with the following paragraph:

The major phosphorylated motifs of neurofilaments are repeated sequences of the type KSPV (SEQ ID NO. 38) where S is the phosphate acceptor; see e.g. Geisler et al., FEBS Lett. 221 (1987), 403-407. Tau has one such motif, centered at S396, and another KSP motif is centered at S235. The two KSP sites lie on either side of the repeat region and are conserved in all tau isoforms. By analogy one may suspect that these sites are involved in the reaction with the SMI antibodies that were raised against neurofilaments. We tested this in three ways, by mutating one or two of the serines, by making smaller tau constructs, and by direct sequencing of tryptic peptides.